Digenic Inheritance of Mutations in the *Coproporphyrinogen Oxidase* and *Protoporphyrinogen Oxidase* Genes in a Unique Type of Porphyria

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The simultaneous dysfunction of two enzymes within the heme biosynthetic pathway in a single patient is rare. Not more than 15 cases have been reported. A woman with a transient episode of severe photosensitivity showed a biochemical porphyrin profile suggestive of hereditary coproporphyria (HCP), whereas some of her relatives had a profile that was suggestive of variegate porphyria (VP). HCP and VP result from a partial enzymatic deficiency of coproporphyrinogen oxidase (CPOX) and protoporphyrinogen oxidase (PPOX), respectively. DNA analysis in the index patient revealed mutations in both the *CPOX* and *PPOX* genes, designated as c.557-15C>G and c.1289dupT, respectively. The *CPOX* mutation leads to a cryptic splice site resulting in retention of 14 nucleotides from intron 1 in the mRNA transcript. Both mutations encode null alleles and were associated with nonsense-mediated mRNA decay. Given the digenic inheritance of these null mutations, coupled with the fact that both HCP and VP can manifest with life-threatening acute neurovisceral attacks, the unusual aspect of this case is a relatively mild clinical phenotype restricted to dermal photosensitivity.

Journal of Investigative Dermatology (2011) **131,** 2249–2254; doi:10.1038/jid.2011.186; published online 7 July 2011

INTRODUCTION

Hereditary coproporphyria (HCP; OMIM 121300) and variegate porphyria (VP; OMIM 176200) are rare autosomal dominantly inherited disorders. Individuals with either HCP or VP may develop photosensitivity, acute neurovisceral attacks, or both (Puy *et al.*, 2010). HCP results from a partial catalytic deficiency of coproporphyrinogen oxidase (CPOX; E.C. 1.3.3.3) and VP from partial deficiency of protoporphyrinogen oxidase (PPOX; E.C. 1.3.3.4), the sixth and seventh enzymes of heme biosynthesis, located in the mitochondrium (Martasek, 1998; Bickers and Frank, 2003; Sassa, 2006; Puy *et al.*, 2010).

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Abbreviations: CPOX, coproporphyrinogen oxidase; HCP, hereditary coproporphyria; PPOX, protoporphyrinogen oxidase; VP, variegate porphyria

Received 30 December 2010; revised 28 March 2011; accepted 7 April 2011; published online 7 July 2011

A decrease in activity of either of these enzymes may lead to a pathological accumulation and measurable excretion of porphyrins and/or porphyrin precursors in urine and stool, and possibly to clinical symptoms. During an acute porphyric attack, a broad spectrum of largely nonspecific and distinct neurovisceral symptoms can manifest, including severe colicky abdominal pain, nausea and vomiting, hypertension, tachycardia, psychiatric symptoms, hemi- and tetraplegia, respiratory failure, and coma (Puy *et al.*, 2010). Attacks may be provoked by several porphyrinogenic factors, such as drugs, alcohol, hormonal changes, infections, and dieting (Bickers and Frank, 2003). In addition to neurovisceral findings, individuals with HCP and VP can also present with cutaneous symptoms on sun-exposed skin, including fragility, blistering, erosions, milia, scars, and post-inflammatory hyperpigmentation (Puy *et al.*, 2010). Another cutaneous feature, facial hypertrichosis, may be observed in VP but rarely in HCP (Bickers and Frank, 2003).

The diagnosis of HCP and VP can be established on the basis of biochemical examination of plasma, urine, and stool samples (Bickers and Frank, 2003). Sometimes, however, these analyses may be inconclusive. This particularly holds true in rare patients with a simultaneous deficiency of two enzymes of heme biosynthesis, referred to as dual porphyria (Poblete Gutierrez *et al.*, 2006). Subsequent determination of the residual enzymatic activity of both enzymes may be

conclusive, but only DNA analysis is confirmative (Bickers and Frank, 2003; Poblete Gutierrez *et al.*, 2006).

In HCP, heterozygosity for mutations in the *CPOX* gene, located on chromosome 3q11.2, results in a ~50% reduction in enzyme activity (Martasek, 1998; Sassa, 2006). Similarly, heterozygosity for mutations in the *PPOX* gene, located on chromosome 1q21–22, results in a 50% reduction in the activity of the encoded enzyme. However, only about 15–20% of mutation carriers for either gene develop clinical symptoms due to incomplete penetrance of the diseases, and symptoms usually do not develop before puberty (Sassa, 2006).

Here, we describe a family in which some individuals showed the biochemical characteristics of HCP, whereas the biochemical abnormalities of others were suggestive of VP. On the basis of additional enzyme activity measurements of PPOX and CPOX in Epstein–Barr virus-transformed lymphoblastoïd cell lines, together with additional molecular genetic studies, we identified a woman in this family who carried mutations in both the *CPOX* and the *PPOX* genes. In spite of carrying mutations in two genes in the heme biosynthetic pathway, the clinical phenotype was restricted to mild dermal photosensitivity.

RESULTS

Biochemical and enzymatic tests

Biochemical examination of urine and feces in the index patient (Figure 1a and individual II-5 in Figure 1b) repeatedly showed an increased urinary excretion of uroporphyrin and coproporphyrin and elevated levels of coproporphyrin and protoporphyrin in the stool, with coproporphyrin concentrations exceeding those of protoporphyrin (Table 1). Results of plasma scanning, plasma aminolevulinic acid and porphobilinogen levels, and CPOX and PPOX activities are also included in Table 1.

Subsequently, several other family members were examined biochemically. Whereas some of these individuals had increased coproporphyrin levels in the feces, another mainly had increased levels of protoporphyrin (Table 1).

DNA analysis

In the index patient, we detected mutations in both the *CPOX* and *PPOX* genes. The *CPOX* mutation is located in intron 1 and consists of a C-to-G transversion 15 bp upstream of the splice-acceptor site, designated as c.557-15C>G (Figure 2a). The *PPOX* mutation is located in exon 12 and consists of a T-insertion at position 1290 of the PPOX complementary DNA, designated c.1289dupT (Figure 2b). The results of DNA analysis in the index patient and the family members tested are depicted in Table 1 and Figure 1b.

Individuals with low PPOX levels carrying the *PPOX* gene mutation were also found in descendants of two of the three paternal great uncles (from two marriages) of the index patient (data not shown).

Reverse transcription-PCR and quantitative real-time PCR

Reverse transcription-PCR showed that the c.557-15C>G mutation creates a cryptic AG splice-acceptor signal that is located 14 bp upstream of the usual splice-acceptor site of intron 1 of the *CPOX* gene (Figure 2c and d).

At the RNA level, both mutation c.557-15C>G in the *CPOX* gene and mutation c.1289dupT in the *PPOX* gene are associated with mRNA decay of 78% and 64%, respectively, compared with expression in a healthy unrelated control (data not shown).

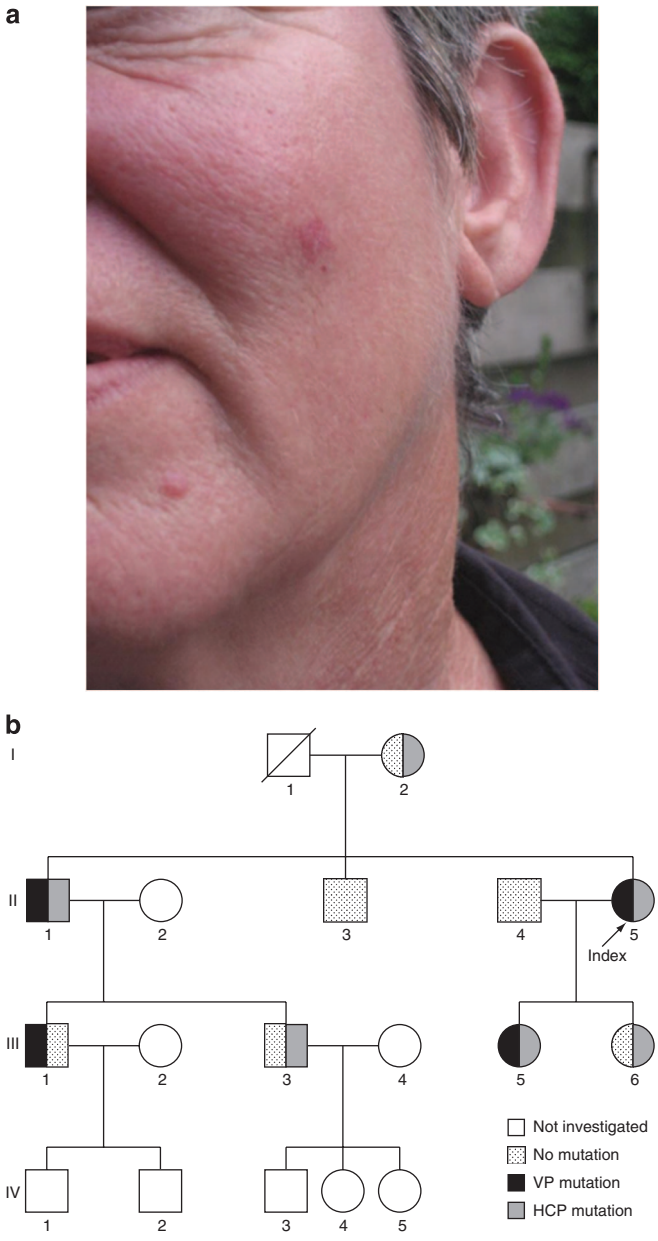

Figure 1. Dutch porphyria family studied. (**a**) Index patient with dual porphyria: note the mild facial hypertrichosis. (**b**) Individuals marked with a dark grey symbol were heterozygotes for the *CPOX* mutation, c.557-15C>G; individuals marked with a black symbol were heterozygotes for the *PPOX* mutation, c.1289dupT; individuals indicated with a white symbol were not investigated; and individuals delineated in speckled grey did not carry a mutation.

Table 1. Biochemical, enzymatic, and molecular genetic profile of the family studied here. Enzymatic activities underlined indicate a decrease below normal levels

Individual	Year of birth	Plasma, ALA <74 nmol l^{-1}	Plasma, PBG <12 nmol l^{-1}	Total plasma, porphyrins <20 nmol l^{-1}	Plasma scanning (semi-quantitative)			PPOX act (N-2sd), >2,200 pmol per mg protein	PPOX mutation	CPOX act (N-2sd), >1,200 pmol per mg protein	CPOX mutation	Urine		Feces		
					Uro	Copro	Proto					Uro (5–36 nmol l^{-1})	Copro (0–230 nmol l^{-1})	Copro (0–30 nmol g^{-1})	Proto (0–135 nmol g^{-1})	
I-1	1910	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	160	774	335	870	
I-2	1910	29	0	6	Neg	Neg	Neg	3,468	—	**876**	c.557-15C>G	0	256	168	90	
II-1	1934	NA	NA	NA	NA	NA	NA	NA	c.1289dupT	NA	c.557-15C>G	100	1,945	469	170	
II-3	1939	42	0	12	Trace	Neg	Neg	2,380	—	1,522	—	0	238	24	82	
II-5	1951	171	53	17	Trace	Trace	Neg	NA	c.1289dupT	NA	c.557-15C>G	198	3,956	989	644	
III-1	1960	NA	NA	NA	NA	NA	NA	NA	c.1289dupT	NA	—	NA	NA	NA	NA	
III-3	1966	NA	NA	NA	NA	NA	NA	NA	—	NA	c.557-15C>G	NA	NA	NA	NA	
III-5	1972	210	58	15	Trace	Pos	Neg	**1,540**	c.1289dupT	**978**	c.557-15C>G	NA	NA	NA	NA	
III-6	1974	118	75	17	Trace	Trace	Neg	2,285	—	**770**	c.557-15C>G	NA	NA	NA	NA	

Abbreviations: ALA, δ-aminovulinic acid; Copro, coproporphyrin; CPOX, coproporphyrinogen oxidase; NA, not available; Neg, negative; PBG, porphobilinogen; Pos, positive; PPOX, protoporphyrinogen oxidase; Proto, protoporhyrin; Uro, uroporphyrin.
Bold and underlined entries emphasize important pathologic deviations.

DISCUSSION

An accurate diagnosis of the different types of porphyria can usually be made through biochemical analyses of urine, stool, erythrocytes, and plasma. These techniques, however, may be insufficient in diagnosing rare patients with dual porphyria (Akagi *et al.*, 2006; Poblete Gutierrez *et al.*, 2006).

The index patient (individual II-5 in Figure 1b) repeatedly showed a biochemical profile suggesting HCP, with fecal coproporphyrin concentrations higher than those of protoporphyrin. When we examined the patient in our outpatient clinic, however, we noted mild facial hypertrichosis (Figure 1a). The cutaneous symptoms in HCP, including skin fragility, vesiculae and bullae, and scarring and hyperpigmentation, cannot be distinguished clinically from those encountered in porphyria cutanea tarda and VP. Nevertheless, skin lesions rarely occur as the only clinical manifestation in HCP and, to the best of our knowledge, there have been no reports of a patient with genetically confirmed HCP with facial hypertrichosis, nor have we seen one in our porphyria outpatient clinic. Therefore, the latter clinical observation in the index patient led to biochemical examination and DNA analysis of the index patient and other family members.

Some family members (individuals I-2, II-1, and II-5 in Figure 1b) had stool porphyrin profiles indicative of HCP, whereas another (individual I-1 in Figure 1b) showed values more compatible with VP (Table 1). The differentiation between HCP and VP may be difficult, mainly because the conditions can present with similar clinical manifestations and overlapping biochemical profiles. In symptomatic patients, plasma scanning can be of diagnostic help because individuals with clinically overt VP usually have a characteristic plasma emission peak at 624–626 nm (Poh-Fitzpatrick, 1980). However, this technique usually fails to identify asymptomatic carriers or children with VP (Da Silva *et al.*, 1995). As enzymatic measurements indicated that some family members might suffer from HCP, whereas others might have VP, we decided to perform DNA analysis in both the *CPOX* and *PPOX* genes in the index patient and eight relatives.

The *CPOX* mutation we found was previously reported in a distant member of this family (Rosipal *et al.*, 1999). We established that this mutation leads to a null allele by introducing a premature termination codon resulting in substantial mRNA decay (Figure 2c and d). The *PPOX* mutation we detected has not been previously described and also generates a null allele associated with mRNA decay.

Until now, 15 patients and families with simultaneous deficiency of two enzymes along the heme biosynthetic pathway have been reported, indicating that dual porphyrias are rare or may be underdiagnosed (Poblete Gutierrez *et al.*, 2006). These results were based mainly on biochemical and enzymatic studies, and only in two instances they were confirmed by molecular genetic analysis (Akagi *et al.*, 2006; Harraway *et al.*, 2006; Poblete Gutierrez *et al.*, 2006). To our knowledge, the constellation of digenic mutations in the *CPOX* and *PPOX* genes has not yet been reported.

In light of the digenic inheritance pattern and the association of both mutations with nonsense-mediated mRNA

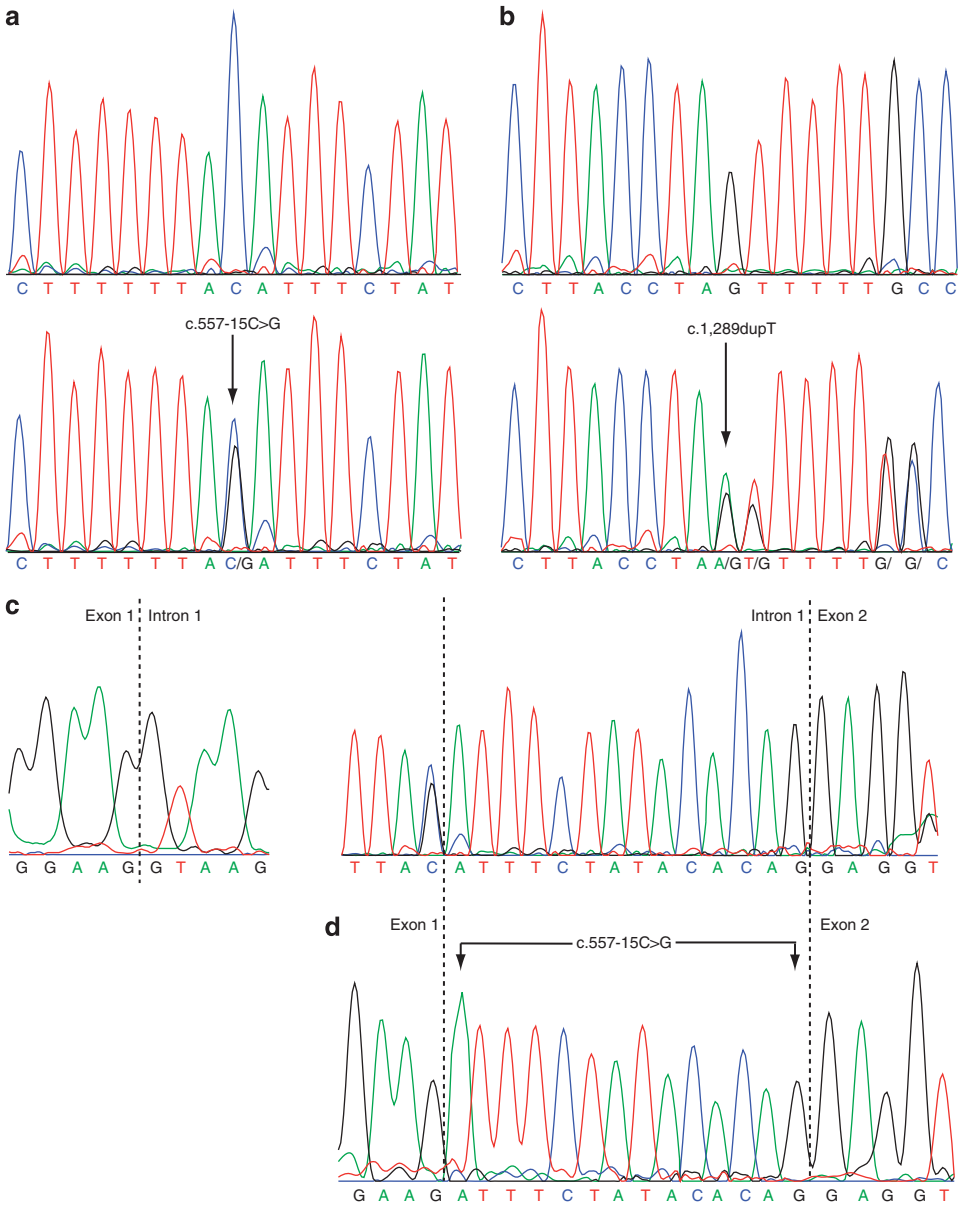

Figure 2. Molecular genetic analysis. (**a**) Mutation of c.557-15C>G in the *coproporphyrinogen oxidase* (*CPOX*) gene (lower panel, indicated by an arrow) compared with the wild-type allele (top panel). (**b**) Mutation of c.1289dupT in the *protoporphyrinogen oxidase* gene (lower panel, indicated by an arrow) compared with the wild-type allele (top panel). Note that the sequence is depicted in reverse orientation. (**c**) Exon 1–intron 1–exon 2 boundaries on the genomic DNA level. (**d**) Cryptically spliced CPOX complementary DNA derived from the mutant allele (cloned from a reverse transcription-PCR product).

decay, we wondered why our patient revealed only mild cutaneous symptoms and had not experienced acute porphyric attacks, in particular, considering the fact that the related patient with mutation c.557-15C>G in the *CPOX* gene reported by Rosipal *et al.* (1999) did present with acute clinical symptoms, comprising abdominal pain, nausea, and vomiting. Considering the turnover rates of the two enzymes involved, perhaps a more severe clinical phenotype might have been expected as a consequence of the two null alleles (Labbe *et al.*, 1985). To the best of our knowledge, the other family members in this pedigree had never experienced overt clinical signs of photosensitivity. Interestingly, the biochemical values of the index patient's brother (individual II-1), who is also heterozygous for mutations in both *CPOX* and *PPOX*, were considerably lower than those found in his sister (Table 1). This finding underlines the fact that in the acute hepatic porphyrias, overt clinical disease is more common in women, most likely due to the association with hormonal status and ingestion of hormonal preparations such as oral contraconceptives. Nevertheless, it should be mentioned that this brother and the father of the index patient were reported with a nonspecific thickening of the facial skin and pronounced facial folds, which could reflect the long-term consequence of previous mild symptoms of cutaneous

porphyria. By contrast, in the two patients with the other genetically confirmed dual porphyrias, the clinical phenotype was heterogeneous, with one patient experiencing only cutaneous symptoms and the other showing signs of an acute porphyric attack (Akagi *et al.*, 2006; Harraway *et al.*, 2006). The heterogeneous clinical phenotypes observed in our index patient and the two individuals with dual porphyria support the current notion that there is little evidence for genotype–phenotype correlations either in the common types of porphyria, which can be inherited in an autosomal dominant or recessive manner, or in the rare patients with dual porphyrias. The notable exception to this rule is autosomal recessive congenital erythropoietic porphyria, in which a certain degree of genotype–phenotype correlation has been described in those individuals carrying the C73R allele (Warner *et al.*, 1992).

In conclusion, we have identified a patient with, to our knowledge, a previously unreported type of dual porphyria, confirmed on the molecular genetic level. We strongly believe that it is important to perform DNA analysis if a dual porphyria is suspected, especially when the biochemical data are not completely conclusive. Molecular genetic analysis for the different types of porphyria is offered by several national porphyria centers throughout Europe (for an overview see http://www.porphyria-europe.com) and, in the United States, by the Department of Human Genetics at The Mount Sinai School of Medicine in New York, NY. Genetic analysis permits an unequivocal diagnosis and also provides affected individuals with the opportunity for genetic counseling. This is an important issue, as an acute neurovisceral porphyric attack can be life threatening.

MATERIALS AND METHODS

Subjects, medical history, and diagnosis

The index patient (individual II-5 in Figure 1b) was a 56-year-old Dutch Caucasian woman who developed severe photosensitivity reactions in her 20s, characterized by blisters, vesicles, and erosions on both hands, the nose, and ears. She was using an oral contraconceptive drug (norethindrone/ethinyl estradiol) and had experienced a cold in the weeks preceding the skin symptoms. After cessation of the oral contraceptive, her symptoms resolved within several weeks. Thereafter, she never again experienced similar symptoms and her further medical history was unremarkable. Her parents were non-consanguineous and the family history was negative with regard to neurological, liver, or kidney disease. When first seen in our clinic, mild facial hypertrichosis was the only abnormal physical finding (Figure 1a).

Biochemical and enzymatic tests

Urine, fecal, and plasma porphyrin scanning was performed as described previously (De Rooij *et al.*, 2003). The use of different eluting solvents and fluorescence scanning permits differentiation of protoporphyrin, coproporphyrin, and uroporphyrin. Plasma aminolevulinic acid and porphobilinogen were measured using enzymatic conversion to uroporphyrin and fluorometric detection. Epstein–Barr-transformed lymphoblastoid cell lines were prepared from peripheral blood by standard methods. CPOX and PPOX activities were measured in these cell lines as described in detail elsewhere (De Rooij *et al.*, 2003).

DNA analysis

After written informed consent was obtained according to guidelines set forth by the local institutional review board and the Declaration of Helsinki Principles, peripheral blood samples from the index patient and eight family members were collected in tubes containing EDTA. Genomic DNA was extracted by following standard techniques (Sambrook *et al.*, 1989). The coding regions and adjacent splice sites of the *CPOX* and *PPOX* gene were amplified by PCR using conditions and primers previously described in detail (Rosipal *et al.*, 1999; Frank *et al.*, 2001). PCR products were subjected to automated sequencing, using an ABI Prism 377 Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequence deviations were detected using the programs Phred, Phrap, and Consed, as described previously (Codon Code Corporation, Dedham, MA; Ewing and Green, 1998; Ewing *et al.*, 1998; Gordon *et al.*, 1998).

Reverse transcription-PCR and quantitative real-time PCR

Total RNA was extracted from peripheral blood mononuclear cells using the RNeasy kit (Qiagen, Venlo, the Netherlands). Complementary DNA was obtained by reverse transcription of total RNA using the Transcriptor High Fidelity cDNA Synthesis Kit with random hexameric primers (Roche Applied Sciences, Almere, the Netherlands). To enable genetic analysis of the *CPOX* transcripts, PCR was performed using primers encompassing exon 2 of the *CPOX* gene. The resulting product was cloned in the pCR-XL-TOPO vector (Invitrogen, Carlsbad, CA) according to the manufacturer's guidelines. A total of 20 clones were selected and, subsequently, the insert was subjected to automated sequencing as described earlier.

mRNA levels for *CPOX* and *PPOX* were measured in RNA specimens from the patient and an age- and gender-matched healthy donor. Therefore, total RNA was derived from whole blood samples using PAXgene Collection Tubes and PAXgene Blood RNA Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Amounts of total RNA were measured using the NanoDrop system (NanoDrop Technologies, Rockland, DE), and equal RNA concentrations of each sample were used for quantitative reverse transcription PCR analysis. TaqMan experiments were conducted on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems) using Assay-on-Demand gene expression products for CPOX (Hs00164367_m1), PPOX (Hs00609392_m1) and 18S (Hs99999901_s1). All measurements were recorded in triplicates in separate reaction wells. The relative mRNA levels were normalized to 18S.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

We are grateful to the patient and her family for their interest and participation in this study. We are also grateful to J.J.A.J. van der Velden for his graphical contributions. J.F. is a board member of the European Porphyria Initiative (EPI) and is, in part, supported by grant number A04155HS, GIS-Institut des Maladies Rares: Network on Rare Diseases to the European Porphyria Initiative (EPI), a grant from the European Union to the European Porphyria Network (EPNET), Program of Community Action in the Field of Public Health, project no. 2006107, and a grant from the Research Committee of the Heinrich-Heine University Düsseldorf, Germany, project no. 9772425. J.M.B. was supported by a grant from the Deutsche Forschungsgemeinschaft (SFB 542, TP C11).

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